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Proteasome Disassembly and Downregulation Is Correlated with Viability during Stationary Phase

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Summary

During prolonged starvation, yeast cells enter a stationary phase (SP) during which the synthesis of many proteins is dramatically decreased [1–3]. We show that a parallel decrease in proteasome-dependent proteolysis also occurs. The reduction in proteolysis is correlated with disassembly of 26S proteasome holoenzymes into their 20S core particle (CP) and 19S regulatory particle (RP) components. Proteasomes are reassembled, and proteolysis resumes prior to cell cycle reentry. Free 20S CPs are found in an autoinhibited state in which the N-terminal tails from neighboring α subunits are anchored by an intricate lattice of interactions blocking the channel that leads into the 20S CPs [4, 5]. By deleting channel gating residues of CP α subunits, we generated an "open channel" proteasome that exhibits faster rates of protein degradation both in vivo and in vitro, indicating that gating contributes to regulation of proteasome activity. This open channel mutant is delayed in outgrowth from SP and cannot survive following prolonged starvation. In summary, we have found that the ubiquitin-proteasome pathway can be subjected to global downregulation, that the proteasome is a target of this regulation, and that proteasome downregulation is linked to survival of SP cells. Maintaining high viability during SP is essential for evolutionary fitness, which may explain the extreme conservation of channel gating residues in eukaryotic proteasomes.

Results and Discussion

Activation of Proteolytic Activity in an Open Channel Mutant

Purified latent 20S CPs can slowly hydrolyze small peptides and some unfolded proteins but cannot degrade multiubiquitinated proteins [5–8]. Previously, deletion of the N-terminal tail of the α3 subunit (*α3ΔN*) resulted in a constitutively active, open channel CP [5]. Proteasomes from *α3ΔN* mutants showed enhanced peptidase activity but not enhanced proteolysis [5, 6]. To test whether the size of the open channel can influence the nature of substrates degraded or the rate of protein degradation, we generated a set of three channel mutants, designated *α3ΔN*, *α7ΔN*, and *α3α7ΔN* , by deleting the conserved N-terminal tails of subunits from opposite sites of the α ring. Purified wild-type (WT) CPs are found in a latent state that can be chemically activated (Figure 1A). Purified CPs from either *α3ΔN* or *α3α7ΔN* strains showed enhanced peptidase activity by an order of magnitude compared to WT. These mutant core particles cannot be further activated by SDS. These results support the conclusion that removal of the α3 tail is sufficient to open a channel by causing disorder in the neighboring tails [5]. Because the α7 tail is peripheral, removing this segment alone may not result in sufficient loss of order in the remaining tails to generate an opening wide enough for small peptides. Thus, peptidase enhancement in the double mutant can be attributed mainly to deletion of the α3 tail.

To study the effect of these mutations on protease activity, casein proteolysis by WT and mutant CPs was measured (Figure 1B). Given that *α3ΔN* core particles are activated for peptidase activity, the rates of proteolysis by WT and *α3ΔN* CPs were remarkably similar (Figure 1B). However, CPs purified from the *α3α7ΔN* strain were significantly more efficient in casein proteolysis. Deletion of two opposing N-terminal tails apparently acts synergistically to relieve hindrance to the entry of proteins into the proteolytic chamber. Our observations suggest that multiple α subunits participate in channel gating. Hereafter, we will refer to the double mutant as the "open channel" mutant. While free CPs do possess some proteolytic capabilities [7–10], these results indicate that, regarding at least some proteins, the CP is found in an inherently latent state and can be activated by channel gating.

Proteasome Gating and Survival during Starvation-Induced Stationary Phase

To understand the biological significance of proteasome gating, it is critical to characterize gating-associated defects at the cellular level. Under favorable conditions, the growth rates of WT and the open channel mutant are indistinguishable (Figure 2A). When nutrients are exhausted, yeast cells enter stationary phase (SP) during which the synthesis of most proteins is attenuated and proliferation ceases [1–3, 11]. During the first week of starvation, both WT and *α3α7ΔN* strains maintain 100% viability (Figure 2B). However, after 10 days in SP, a precipitous drop in viability of mutant cells was observed. In contrast, WT cells are viable even after lengthy periods of starvation.

After 6 days of starvation, when viabilities of WT and *α3α7ΔN* strains are still indistinguishable, outgrowth rates from SP differ. The *α3α7ΔN* strain shows a significantly delayed exit from SP of ~13 hr, versus a 6-hr delay for WT (Figure 2C). In addition, the subsequent growth rate of the mutant is initially slower than that of WT. Apparently, the open channel mutant gradually deteriorates during SP; it first displays delays in resump-

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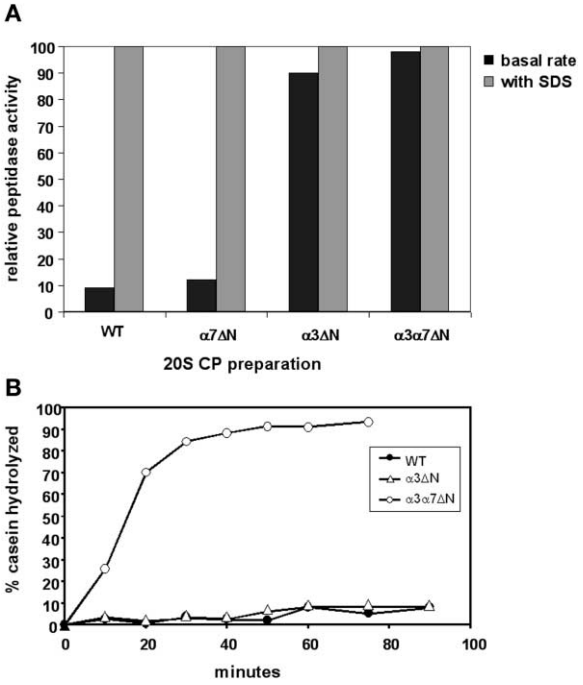

Figure 1. Enzymatic Properties of 20S Core Particles Purified from WT or N-Terminally Truncated α Subunit Mutants

A set of three channel mutants was generated by deleting the highly conserved N-terminal tails of two subunits from opposite sites of the α ring. Nine residues (GSRRYDSRT) forming the extreme N terminus of the α3 subunit were deleted in *α3ΔN*. In a second mutant, *α7ΔN*, ten residues (TSIGTGYDLS) were truncated from α7. Finally, a double mutant, *α3α7ΔN*, had both truncations.

(A) Relative peptidase rates of 20S CPs purified from WT, *α3ΔN*, *α7ΔN* , and *α3α7ΔN* strains (left bar in each pair) and in the presence of 0.02% SDS (right bars). Purified WT CPs are found in a latent state that can be activated by 0.02% SDS, while *α3ΔN* is at near maximal peptidase activity. Deletion of the α7 N terminus alone does not significantly enhance peptidase activity as compared to WT. Enzyme activity was measured as arbitrary fluorescence units of the liberated 7-amido-4-methylcoumarin (AMC) from the test substrate suc-LLVY-AMC.

(B) Proteolysis by 20S CPs. ^{14}C-labeled casein was incubated with identical amounts of purified 20S core particles from WT, *α3ΔN*, and *α3α7ΔN* strains. The undegraded substrate was TCA precipitated, and soluble radioactivity, representing peptide products from proteolyzed protein, was measured.

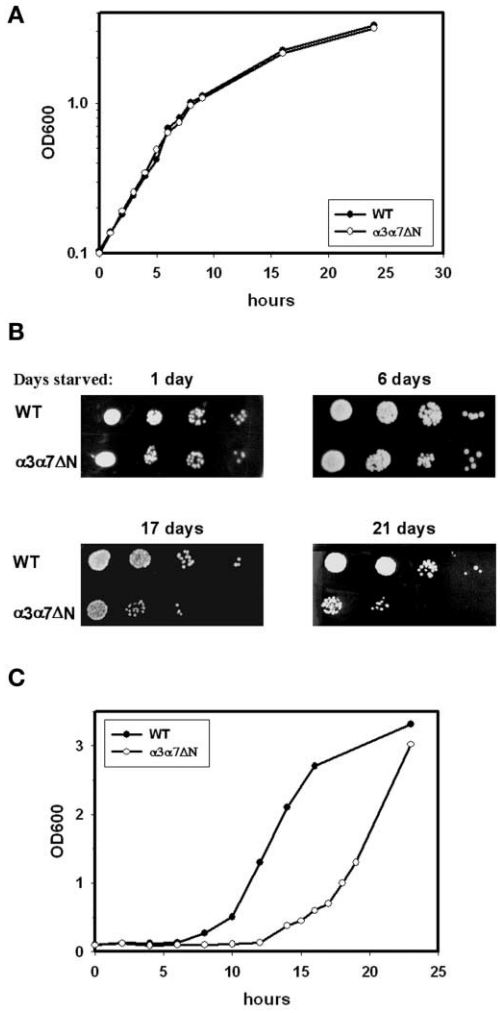

Figure 2. Growth Rates and Viability of WT and an Open Channel Mutant

(A) Log phase. Logarithmically growing cultures (Log) of WT and *α3α7ΔN* were diluted into YPD (yeast extract, peptone, dextrose)-rich medium, and cell density was measured over time.

(B) Viability. Liquid culture was kept aerated at 30°C for 3 weeks without the addition of nutrients. The total number of cells in the culture was counted by microscopy and did not change significantly over the duration of the experiment. The number of viable cells was tested daily by serial dilution onto YPD-agar during the starvation period indicated. Viability was calculated from the number of colonies formed compared to the total number of cells.

(C) Outgrowth from SP. Cultures of WT *α3ΔN* and *α3α7ΔN* yeast were kept in stationary condition for 6 days, then diluted in YPD medium; resumption of growth was measured with time. As shown in (B), viability levels of WT and an open channel mutant after 6 days of starvation are identical.

tion of growth and then eventually dies. Maintaining high viability rates during SP, as well as the ability to rapidly resume normal cell growth and division upon contact with nutrients, is vital for evolutionary fitness, possibly explaining the high level of sequence conservation of gating residues.

Inhibition of the Proteasome during Stationary Phase

To ascertain the link between proteasome activity and endurance during starvation, we measured in vivo proteolysis rates in WT and the open channel mutant. Intracellular proteolysis was measured by pulse-chase analysis in Log phase as well as following prolonged starvation. We chose short-lived substrates that are ubiquitinated by different sets of ubiquitin-conjugating and ubiquitin-ligating enzymes: Ub-Tyr-β-galactosidase is ubiquitinated by the N-end rule pathway, while Ub-Pro-β-galactosidase is tagged by the UFD pathway enzymes [12, 13]. The half-lives of these substrates are long enough to enable observation of small increases in degradation rates within experimental error.

During logarithmic growth (Log), both substrates were degraded more rapidly in *α3α7ΔN* cells than in WT (Figure 3A). Our observation that open channel mutations

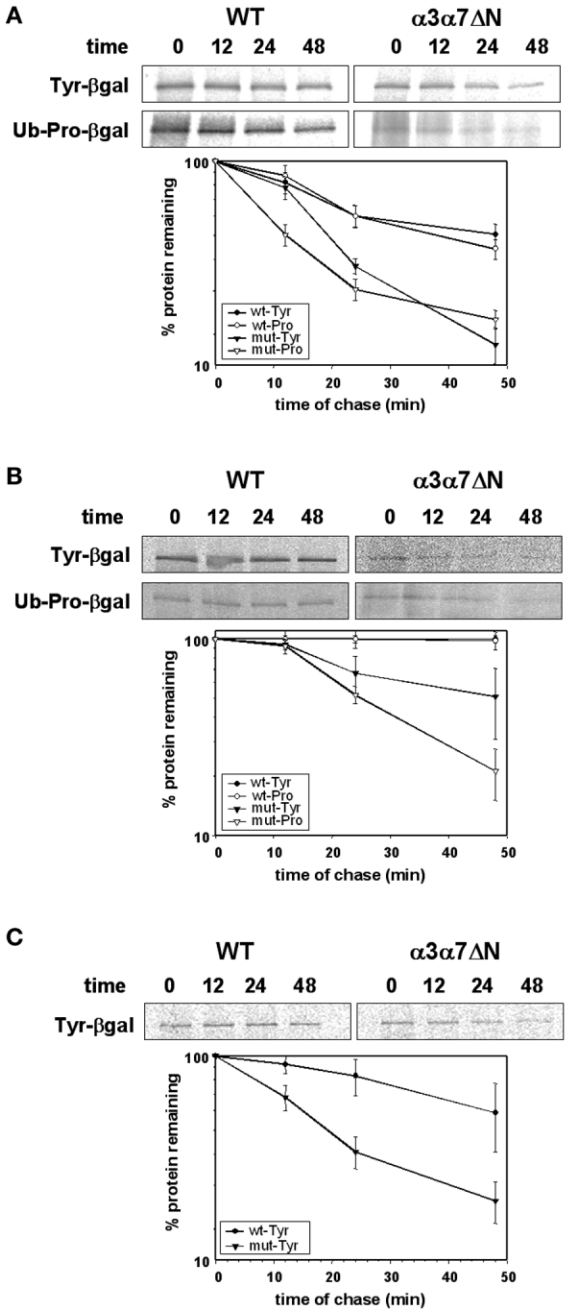

Figure 3. In Vivo Degradation Rates of Short-Lived Proteins in WT and an Open Channel Mutant

Pulse-chase analysis was performed on N-end rule or UFD pathway substrates expressed in WT (circles) and $\alpha 3\alpha 7\Delta N$ (triangles). The error bars reflect the average of three independent experiments, an example of which is shown above each graph.

(A) Log. Degradation rate of Tyr-β-galactosidase (filled circles) and Pro-β-galactosidase (hollow circles) in logarithmic cells.

(B) SP. Yeast cultures were kept aerated at 30°C for 6 days without the addition of nutrients. Cells were diluted into appropriate media, and pulse-chase analysis was performed. Note that radiolabeling of substrates in $\alpha 3\alpha 7\Delta N$ cells is less efficient than in WT following prolonged starvation, probably because proteolysis during SP influences multiple metabolic pathways.

(C) Exit from SP. Degradation of Tyr-β-galactosidase in cells starved for 6 days was tested 5 hr after dilution and addition of nutrients to stationary cells. Proteolysis is restored to near normal levels prior to resumption of cell growth and proliferation (Figure 2C).

accelerate proteolysis of bona fide substrates of the ubiquitin pathway in vivo indicates that the proteasome itself must be partially rate limiting in the overall process of protein removal and that channel gating plays a regulatory role in proteasome function. The open channel apparently accelerates protein degradation by relieving hindrance on substrate translocation into the proteolytic chamber of the CP. That mutant cells do not exhibit a growth phenotype during Log most likely reflects efficient replacement of proteins that does not occur in SP.

Degradation rates of both test substrates were severely inhibited in starved WT cells (Figure 3B). SP is accompanied by a general decrease in the levels of transcription and translation [1, 2, 14]. Stabilization of certain proteins during the Log to SP transition has also been inferred by studying their steady-state levels [15]. Based on the results of Figure 3, the proteasome pathway is in fact downregulated in starved, growth-arrested cells. This is a remarkable correlation of proteasome disassembly and inhibition with regulatory purposes. Interestingly, the difference in the proteolytic behavior of WT and the open channel mutant is enhanced during starvation (Figure 3B). Proteolysis of Ub-β-galactosidase fusions persists during SP in the open channel mutant; this finding indicates that the proteasome is a target for this proteolytic downregulation. Upon exit from SP, the inhibition of proteasome function imposed in starved cells is relieved and proteolysis resumes (Figure 3C).

Disassembly of 26S in Stationary Phase as an Inhibitory Mechanism

The decrease in proteolysis during SP could result from proteasome inhibition or a decrease in proteasome levels. To elucidate this point, we monitored proteasome levels by nondenaturing electrophoresis on crude extracts from WT and $\alpha 3\alpha 7\Delta N$ cells (Figure 4A). Interestingly, in growing cells (Log), proteasomes are found almost exclusively in the 26S holoenzyme state. A gradual decrease in 26S holoenzyme levels is observed throughout SP and is accompanied by an increase in levels of free CP; thus, differing growth conditions may alter the assembly state of native proteasomes [16, 17]. Immunoblotting for 19S RP subunits shows no significant change in their levels throughout SP; this finding confirms that proteasomes disassemble into stable components (Figure 4A).

To estimate the overall levels of unassembled proteasome components in starved cells, we tested proteasome reassembly both in vitro and in vivo. Incubation of extracts from starved cells with high levels of ATP allowed for 26S holoenzyme formation, pointing to stable levels of 26S components (Figure 4B). Similar reassembly of proteasomes occurs naturally upon exit from SP, within 1 hr of nutrient addition (Figure 4B). Proteasome components are thus apparently present during SP and can reassemble upon exit from SP, possibly for removal of SP-specific inhibitors of metabolic pathways. Interestingly, outgrowth of the open channel mutant is delayed, even though proteasome reassembly occurs similarly to WT. Sustained proteolysis observed in this mutant during SP (Figure 3B) may have deleterious consequences, necessitating a longer adaptation process (Figure 2C).

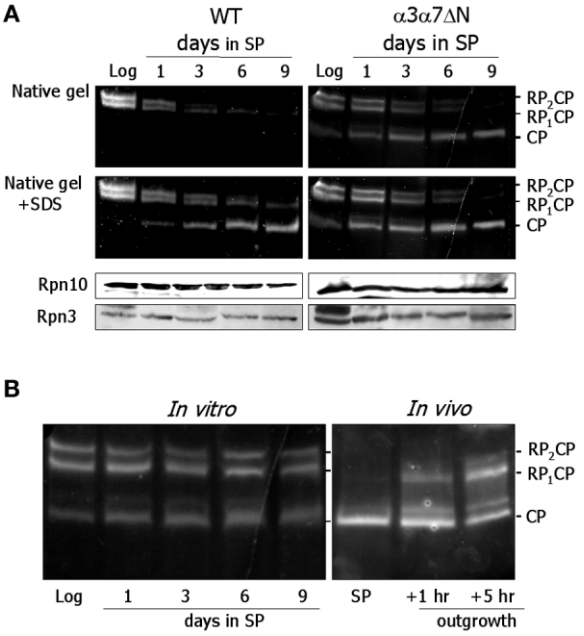

Figure 4. Changes in Proteasome Assembly State in Mature Stationary Phase

Nondenaturing gel electrophoresis of holoenzymes was followed by in-gel peptidase activity assay. Symmetric (RP_2CP) and asymmetric (RP_1CP) forms of the holoenzyme as well as the CP form are indicated.

(A) Crude extract of WT (left) and *α3α7ΔN* (right) was prepared from rapidly growing cells (Log) and at different stages of starvation (SP). The activity of free WT core particles is naturally repressed but can be easily visualized upon activation with small amounts of SDS (middle panels). The levels of the 26S holoenzyme, the predominant state of the proteasome in dividing yeast cells, decrease during SP. The relative abundance of 19S components during this time was monitored by immunoblotting (bottom panels). Proteasomes stability in the open channel mutant is similar to WT.

(B) Proteasome reassembly. Crude whole cell extract from Log and starved mutant cells was prepared as in (A) and was incubated for 1 hr with 5 mM ATP. Proteasome holoenzymes are visualized by nondenaturing gels, indicting that similar levels of proteasome components are present throughout SP (left panel). Native gels of rapidly prepared crude cell extract from stationary cultures during exit from SP show reassembly of proteasome holoenzymes in vivo within 1 hr of nutrient addition and prior to resumption of cell proliferation (right panel). Similar results were obtained with WT extracts.

The Proteasome in Growth and Starvation

While biosynthetic processes are generally downregulated in SP, prior studies have pointed to enhanced protein degradation during adaptation to nutrient deprivation. First, the vacuolar/lysosomal machinery, which mediates autophagy, is enhanced under these conditions. Indeed, upon starvation, the bulk of proteolysis takes place in the vacuole, probably to allow for recycling of cytosolic material, explaining why mutants in vacuolar proteases rapidly lose viability [18–20]. Also induced during the transition to SP are components of the ubiquitin-proteasome pathway [14, 21, 22]. However, expression of these genes later subsides [14]. In synchrony, proteasome-dependent proteolysis is enhanced during early SP [21] and is then suppressed during late, or mature, SP (Figure 3). Thus, the proteasome probably does not contribute significantly to bulk proteolysis in mature SP [18, 19].

We have found that downregulation of proteasome activity is a novel regulatory response in starved and growth-arrested cells, indicating that reversible inhibition and reactivation of the proteasome can be harnessed adaptively. While the natural state of the proteasome in rapidly growing yeast is the 26S holoenzyme, levels gradually decrease until the autoinhibited 20S CP is the dominant form in stationary cells (Figure 4). Whether proteasome activity at some much-reduced level remains required in mature stationary phase cells may be testable by using reversible proteasome inhibitors. This mode of regulation is highly unexpected, as, usually, the focus is on E3 ubiquitin ligases as regulators of the pathway [23]. Downregulation of other components of the ubiquitination pathway may occur during SP as well.

The presence of 26S proteasome components, free RPs and CPs, in starved cells raises an intriguing question: are proteasomes disassembled into inherently inactive subcomplexes as a mechanism to inhibit proteolysis, or do these entities play independent roles? In vitro, free CPs possess the ability to hydrolyze certain proteins independently of ubiquitin and ATP [7], while free RPs can refold some misfolded proteins [24, 25]. The deleterious effects of the activated open channel 20S CP present upon proteasome disassembly, as described in this work, is in accord with these activities of the proteasome subcomplexes. Thus, the balance between degradation and refolding may be adjustable based on the availability of resources and physiological needs.

Gating appears to regulate proteasome function in several ways. We have suggested that gating residues impose inhibition on free CPs ([5] and Figure 1). Open channel mutations accelerate in vivo proteolysis of bona fide substrates of the ubiquitin pathway; this finding indicates that the 26S proteasome must be at least partially rate limiting (Figure 3). That channel opening is a decisive factor in facilitating degradation explains the extraordinary conservation of residues that gate this channel. Stationary phase has many features in common with G_0 in mammalian cells; therefore, the regulatory mechanisms described in the present work may provide insight into the mammalian cell cycle as well.

Supplemental Data

Supplemental Data including the Experimental Procedures are available at http://www.current-biology.com/content/supplemental.

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